Enantiomeric separation of zopiclone, its metabolites and products of degradation on a β-cyclodextrin bonded phase

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Abstract

In this paper the separation of zopiclone (ZP) enantiomers, its degradation products and chiral metabolites, has been investigated on a β-cyclodextrin bonded phase. The liquid chromatographic behavior of the enantiomers of zopiclone, zopiclone-N-oxide (OXZP) and N-desmethyl-zopiclone (DMZP) has been studied with respect to mobile-phase composition, pH, ionic strength and the nature of the organic modifier. Based on the results, conditions were chosen for the separation of the enantiomers of zopiclone, its chiral metabolites and its related degradation products. The separation selectivity of a β-cyclodextrin (β-CD) bonded-phase column was examined. Further, based on crystallographic data, a computer model of the ZP molecule has been designed by using the HyperChem program in order to estimate the possibility of the different groups to interact with the β-CD and to give a plausible explanation of the mechanism of chiral discrimination.

Keywords: Enantiomer separation; Pharmaceutical analysis; Optimization; Mobile-phase composition; Zopiclone; Cyclodextrins

1. Introduction

The chiral zopiclone, 6-(5-chloro-2-pyridyl)-5-(4-methyl-1-piperazinyl)carbonyloxy-7-oxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazine (RP 27267) (Fig. 1), is the first compound of the cyclopyrrolone class of hypnotics and it is marketed as a racemate. It is structurally unrelated to the benzodiazepine derivatives (BZD); nevertheless, it acts via the benzodiazepine/GABA receptor complex although through a different mode of action than BZD. It is indicated for the short-term treatment of insomnia, transient,

situational or chronic insomnia, insomnia secondary to psychiatric disturbances [1–4].

Several pharmacokinetic studies have been performed which showed that, after oral administration, zopiclone (ZP) is well and rapidly absorbed with a bioavailability of 80% [5]. It is metabolized by three major pathways involving decarboxylation, oxidation and demethylation. (About 50% of the dose is hydrolysed and decarboxylated to unknown metabolites.) The main metabolites in urine are the less active zopiclone-N-oxide (OXZP) (RP 29753) and the inactive N-desmethyl-zopiclone (DMZP) (RP 32273). During the metabolism of ZP the asymmetric carbon on the pyrazine group in position 2 remains and consequently, the two metabolites

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Zopiclone
(RP 27267)

RP 29307
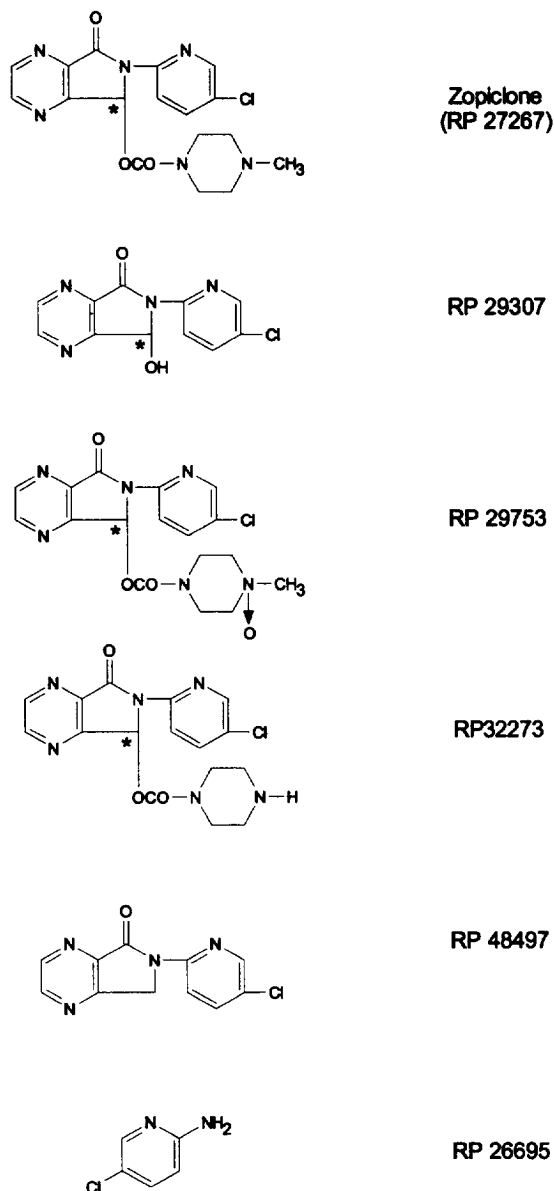
RP 29753

RP32273

RP 48497

RP 26695

Fig. 1. Chemical structures of ZP (RP 27267), RP 29307, OXZP (RP 29753), DMZP (RP 32273), RP 48497, RP 26695.

OXZP and DMZP are also chiral. Recent studies demonstrate that the pharmacokinetics of zopiclone might be stereoselective. Absorption is probably not involved in this stereoselectivity while metabolism is presumably the step responsible for this specificity [6,7].

Chromatographic methods have been described in the literature for the determination of the racemates

of ZP and its metabolites. Three different types of chiral stationary phases have been utilized for the separation of the enantiomers of ZP, OXZP and DMZP: a poly-methacryloyl-(1R,2S)-norephedrine stationary phase [6], an ovomucoid column [6,8,9] and cellulose carbamate columns [8–11]. Moreover, recently a capillary electrophoresis method has been developed for the enantiomeric resolution of these racemic mixtures [7].

Further, many techniques have been reported for the determination of zopiclone [12–15] and its potential impurities (Fig. 1). It is important to mention that some of these impurities are also chiral and their polarities are very different [16]. The chiral OXZP can also be considered as an impurity of ZP and is formed by oxidation. The chiral related substance RP 29307 is formed during the penultimate phase of synthesis. It may pre-exist in the starting material, but it can also be formed by hydrolysis of zopiclone during storage. RP 26695 does not pre-exist in the starting material but it is an achiral potential product of degradation during storage. Finally, RP 48497 is an achiral potential photo-degradation product caused by bright light.

In this paper, a direct chromatographic method has been developed in order to investigate the separation of the enantiomers of zopiclone, zopiclone-N-oxide (OXZP) and N-desmethyl-zopiclone (DMZP), as well as its potential degradation products by using a β-cyclodextrin bonded phase [17].

Cyclodextrins (CDs) are toroidally shaped oligosaccharides composed of glycopyranose units bonded together via α-(1,4)-linkages [18]. By virtue of their structures, CDs create reversible, encapsulating environments into which various organic and inorganic molecules can enter and orient themselves allowing highly specific interactions.

The fundamental mechanism of the formation of inclusion complexes between the host and guest molecules involves interaction between the hydrophobic cavity of the CD and the lipophilic portions of the analyte. However, this interaction may be the result of a variety of other interactions such as hydrogen bonding with the –OH groups at the periphery of the cavity, the release of high-energy water or organic modifier during complex formation, or a combination of the above factors. On the other hand, solvent interaction plays a competitive role and

it is preferable that the solute has a greater affinity for the binding site (CD cavity) than the organic modifier [19].

The liquid chromatographic behavior of the enantiomers of zopiclone, OXZP, DMZP and of the related substances (RP 29307, RP 26695, RP 48497) has been studied with respect to mobile-phase composition, pH, ionic strength and the nature of the organic modifier. Based on the findings of this work, conditions were chosen for the simultaneous enantiomeric resolution of zopiclone and its metabolites in the presence of the related degradation products.

Finally, based on crystallographic data [20] a computer model of the ZP molecule has been designed by using the HyperChem program. The aim of this study was to estimate the possibility of the different groups to interact with the β-CD in order to give a plausible explanation of the mechanism of chiral discrimination.

2. Experimental

2.1. Apparatus

The liquid chromatographic system consisted of a Waters Model 501 pump, a Rheodyne Model 7125 injector with a 5-μl loop, and a Waters Model 486 UV–Vis detector with an 8-μl flow cell. The chromatograms were obtained by using a Hewlett-Packard Model HP3394A integrator.

A LiChroCART 250×4 mm I.D. ChiraDex (5 μm) column was obtained from E. Merck (Darmstadt, Germany). When not in use the column was stored in methanol–water (40:60); the pH was measured with a Metrohm Model 654 Herisau pH meter .

All experiments were performed at room temperature (about 25°C). Detection was accomplished at a wavelength of 305 nm.

2.2. Chromatographic conditions

Buffers were prepared using triethylamine solutions of different concentrations (i.e., 0.1–1.5%) which were adjusted by addition of glacial acetic acid to the desired pH (i.e., 3.5–7.2). The mobile phases, consisting of triethylammonium acetate buffer and the appropriate amount of organic modifier, were freshly prepared, filtered through a 0.45-μm Millipore filter and degassed under vacuum using a Millipore system.

The effect of the organic modifier on the separation of the above-mentioned compounds was examined with binary mobile-phase systems and was based on the findings of previous work [21].

2.3. Solutions

The stock standard solutions of all compounds (1.00 mg/ml) were accurately prepared by dissolving an appropriate amount of the compound in HPLC-grade water or methanol; the standard solutions were kept in amber bottles in the refrigerator and renewed every week. Working standard solutions of the compounds under study (5.0 μg/ml) were prepared every day in mobile phase. Typically, a volume of 5 μl of each solution was injected. The void volume of the column was determined by injecting 5 μl of pure methanol.

To evaluate the reproducibility of the retention times, each run was triplicated.

2.4. Materials

All solvents were of HPLC grade and were purchased from Lab-Scan. Triethylamine and glacial acetic acid were of analytical grade and were purchased from Aldrich. Pure substances (in the form of hydrochloride salts) of the racemic mixtures of zopiclone (RP 27267), OXZP (RP 29753), DMZP (RP 32273), and of the related compounds RP 29307, RP 48497, RP 26695, were kindly provided by Rhône-Poulenc Rorer and were used without further purification.

3. Results and discussion

3.1. Effect of mobile-phase composition and solvent selectivity

The effect of the mobile-phase composition on the retention (t_R) and resolution (R_s) of the enantiomers of ZP, OXZP and DMZP, as well as of RP 29307, RP 48497 and RP26695 was investigated by changing

the organic modifier-to-buffer ratio from 5:95 to
60:40. It was observed that the dependence of log k'
of each compound on the content of the organic
modifier in the mobile phase was mostly linear,
suggesting a reversed-phase mechanism for the
interaction between solutes and stationary phase
[22]; thus, the inclusion process seems to be the
predominant factor for their retention.

Two different organic modifiers have been used,
i.e., acetonitrile (ACN) and methanol (MeOH),
based on the findings of a previous study [21]. In
that study it was of interest to estimate the role of
solvent selectivity in the chiral discrimination pro-
cess by using binary (aqueous/organic) mobile-phase
systems which had the same polarity index. Par-
ticularly with MeOH and ACN a very good sepa-
ration was achieved for the enantiomers of zopi-
clone. Thus, in this study MeOH and ACN were
selected for the investigation of the direct enantio-
meric resolution of ZP, OXZP and DMZP in the
presence of zopiclone's potential impurities.

The use of both solvents leads to a successful and
rapid separation of ZP enantiomers. However, in
further studies it was shown that ACN was needed to
achieve the simultaneous enantiomeric resolution of
OXZP and DMZP.

Thus, after the above-mentioned preliminary
studies two mobile-phase compositions, i.e., 90:10 or
95:5 buffer–organic modifier, were chosen to pro-

vide the optimum chromatographic conditions for the
investigation of the other chromatographic parame-
ters.

3.2. Effect of ionic strength

The effect of ionic strength on the separation of
the compounds under study was investigated by
varying the concentration of triethylammonium ace-
tate (TEAA) from 0.1% to 1.5% (w/v) at different
pH values.

As shown in Fig. 2, the retention time of the
enantiomers of ZP decreases as the electrolyte
concentration increases. This behavior can be
rationalized in terms of triethylammonium acetate
molecules being included in the β-CD cavity, thus
competing with the solute molecules. This mecha-
nism is possible through the formation of a tri-
ethylammonium acetate ion pair within the cavity.
An alternative mechanism may be that the tri-
ethylammonium acetate molecules interact with the
hydrophilic secondary hydroxyl groups at the rim of
the cavity, thus blocking the entry of the analytes
[22].

A concentration of 0.5% (w/v) triethylammonium
acetate (TEAA) was chosen as the optimum con-
centration for the simultaneous separation of the
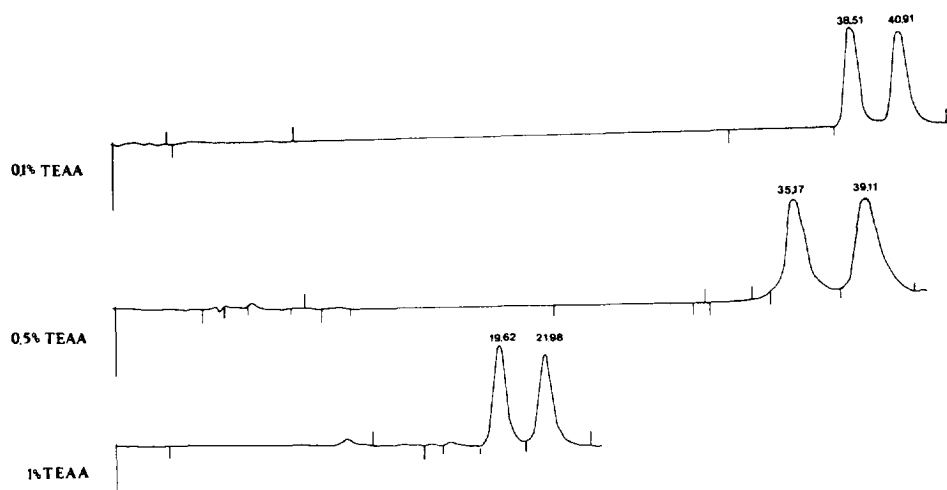enantiomers and the compounds under study.

Fig. 2. Effect of TEAA concentration on the enantiomeric resolution of ZP. Chromatographic conditions: buffer–ACN, 95:5; pH 7.2;
flow-rate, 0.5 ml/min.

Table 1
Effect of pH on the separation of ZP enantiomers by using ACN as organic modifier[a]

pH	t_{R1} (min)	t_{R2} (min)	R_s
3.5	5.68	–	–
4.5	6.67	7.25	0.48
5.5	9.52	10.59	1.05
6.7	24.80	27.63	1.66
7.2	35.17	39.11	1.79

[a] Chromatographic conditions: buffer–ACN 95:5; buffer, 0.5% TEAA (w/v); flow-rate, 0.5 ml/min.

3.3. The effect of pH

The effect of pH on the retention and resolution of the compounds under investigation was studied by changing the pH of the aqueous component of the mobile phase from 3.50 to 7.20 (respecting column limitations).

Two different organic modifiers, i.e., acetonitrile and methanol, have been used, each of which exhibits a different behavior on retention and resolution. Table 1 and Table 2 show the effect of pH on the retention time and the resolution of ZP enantiomers using ACN and MeOH, respectively, as organic modifier.

As discussed earlier, inclusion complex formation has been suggested as the predominant mode of retention. Hydrophobic interactions between the β-CD cavity and the enantiomers of ZP, as well as hydrogen bonding interactions with the –OH groups in the circumference of the β-CD molecules are strongly affected by pH due to the presence of ionizable groups in the molecule.

Zopiclone has three distinct groups which can be protonated by decreasing the pH of the mobile phase,

Table 2
Effect of pH on the separation of ZP enantiomers by using MeOH as organic modifier[a]

pH	t_{R1} (min)	t_{R2} (min)	R_s
4.5	5.81	6.26	0.22
5.0	6.68	7.40	0.36
6.0	9.90	11.37	0.98
6.5	12.48	14.46	1.13
7.0	14.73	17.63	1.66

[a] Chromatographic conditions: buffer–MeOH 90:10; buffer, 1% TEAA (w/v); flow-rate, 0.8 ml/min.

i.e., the piperazinyl nitrogen atom ($N-CH_3$), the pyridyl nitrogen atom and the pyrazinyl nitrogen atom (Fig. 1). It has been reported that from a pharmacological point of view it is of interest to study the acid–base equilibria involved in the protonation of the nitrogen atoms in the zopiclone molecule. The first protonation is that of the $N-CH_3$ piperazinyl atom, and it was apparent that protonation of the piperazinyl nitrogen atom is associated with very little spectral changes. This is because the lone-pair electrons of the piperazinyl nitrogen atom are not engaged in the resonance of the chromophoric group [23]. Thus, the pK_a value of this group has been determined potentiometrically, i.e., pK_a 6.7 [16].

With the pyridyl nitrogen atom being more basic than the pyrazinyl nitrogen atom, the second protonation concerns the pyridyl nitrogen atom; this leads to a change of the UV spectrum of zopiclone; this UV spectral change permits the determination of the pK_a value which was found to be -1.5 for the pyridinium–pyridine equilibrium. Further increase of pH did not give spectral changes [23].

Based on the above it is evident that in the pH range examined in this study the only protonation which occurs is that of the piperazinyl nitrogen atom (pK_a 6.7). This protonation strongly affects the inclusion process of ZP enantiomers in the β-CD cavity; this is expressed by a dramatic increase in the retention time at pH equal or greater than 6.7.

At pH between 3.5 and 5.5 the enantiomers of ZP are expected to be mostly in their ionic form; thus, retention depends to a great extent on the ion-pair formation between the charged nitrogen group and the acetate anion, the degree of which decreases with increasing pH, and consequently, an increase in retention time is observed.

At pH equal or greater than 6.7 more than 50% of the ZP enantiomers are expected to be unprotonated resulting in a variation of the stability of their inclusion complexes within the β-CD cavity, which reflects the respective variations in the stability constants (K_f) of the complexes, and a dramatic increase of their retention. It is generally accepted that the uncharged compounds tend to form more stable complexes within the hydrophobic CD cavity than the charged solutes.

Table 3 shows the dependence of the retention

Table 3
Equations relating retention (t_R) of zopiclone enantiomers[a] with pH, using (A) ACN or (B) MeOH as organic modifier

a	b	r	n
(A) $1/t = a + b$ pH			
0.332 (±0.016)	−0.0424 (±0.0029)	0.993	5
0.325 (±0.0088)	−0.0422 (±0.0016)	0.998	5
(B) $t = a$ pH[b]			
−1.52 (±0.22)[b]	2.15 (±0.12)	0.994	5
−1.77 (±0.22)[b]	2.37 (±0.12)	0.996	5

[a] Pure enantiomers were not available.
[b] The intercept is equal to log a.

time of ZP enantiomers on pH by using ACN (A) and MeOH (B) as organic modifier and Fig. 3 presents the regression analysis plots representing the best fitting of the data.

By increasing the pH, the solutes become more retained and hence column's inclusion selectivity also increases. Thus, it could be expected that the enantiomeric resolution (R_s) of ZP, would also increase by increasing pH.

There is a linear relationship between the enantiomeric resolution R_s and pH when using ACN as organic modifier (Table 4, A), whereas a multiplicative model is proposed to describe the dependence of R_s on pH using MeOH as organic modifier (Table 4, B); Fig. 4 shows the regression analysis plots providing the best fitting of the data.

The retention of the other compounds under study is not so dramatically affected by a change in pH. An increase in retention time is also observed for the enantiomers of OXZP and DMZP and for RP 29307, RP 48497 and RP26695, as shown in Fig. 5. It important to note that at pH less than 7.2 there is considerable overlapping in the separation of the
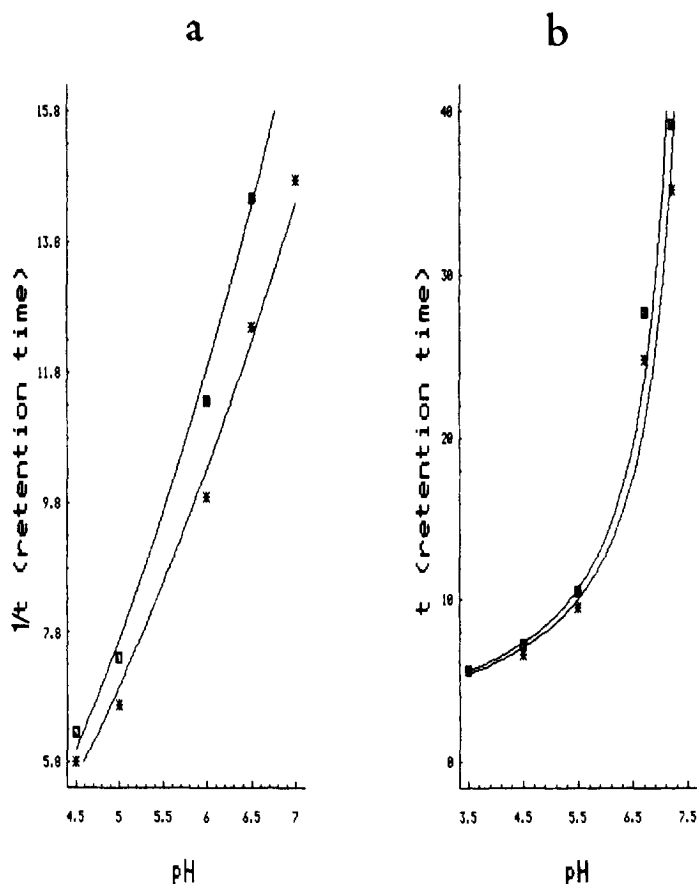

Fig. 3. Dependence of retention (t_R) of ZP enantiomers on pH by using ACN (a) and MeOH (b) as organic modifier.

Table 4
Equations relating resolution (R_s) of zopiclone enantiomers with pH, using ACN (A) or MeOH (B) as organic modifier

a	b	r	n
(A) $R = a + b$ pH			
−1.74 (±0.11)	−0.499 (±0.019)	0.998	5
(B) $R = a$ pH^b			
−8.31 (±0.47)[a]	4.54 (±0.27)	0.995	5

[a] The intercept is equal to log a.

second enantiomer of OXZP and the first enantiomer of DMZP; thus, it seems that the optimum pH for the simultaneous separation of the enantiomers of ZP, OXZP and DMZP, in the presence of the potential impurities RP 29307, RP 48497 and RP26695, is 7.2.

3.4. Evaluation of elution order

The hypothesis derived in this work accounts for the reversed-phase retention behavior of ZP, OXZP and DMZP enantiomers and RP 29307, RP 48497, RP26695 related substances using a β-CD column; furthermore, it suggests a possible explanation for the mechanism of chiral discrimination.

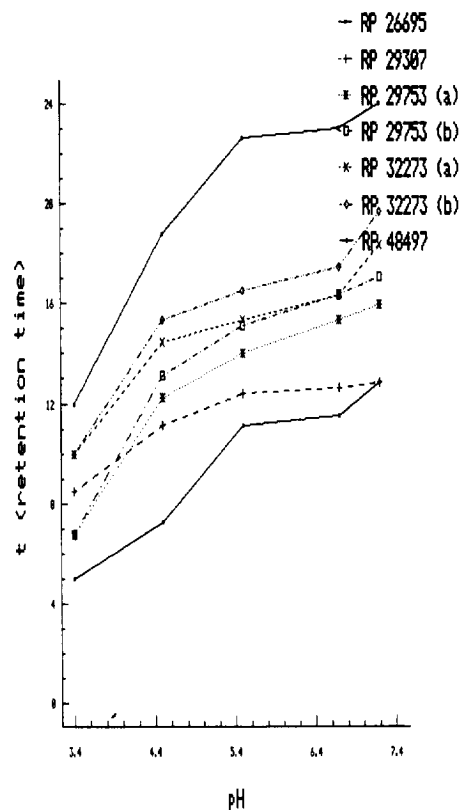Generally, the formation of an inclusion complex

Fig. 5. Dependence of retention (t_R) of the enantiomers of OXZP, DMZP (pure enantiomers were not available) and of the related substances RP 29307, RP 48497, RP 26695, on pH. Chromatographic conditions: buffer–ACN 95:5; buffer, 0.5% TEAA (w/v); flow-rate, 0.5 ml/min.
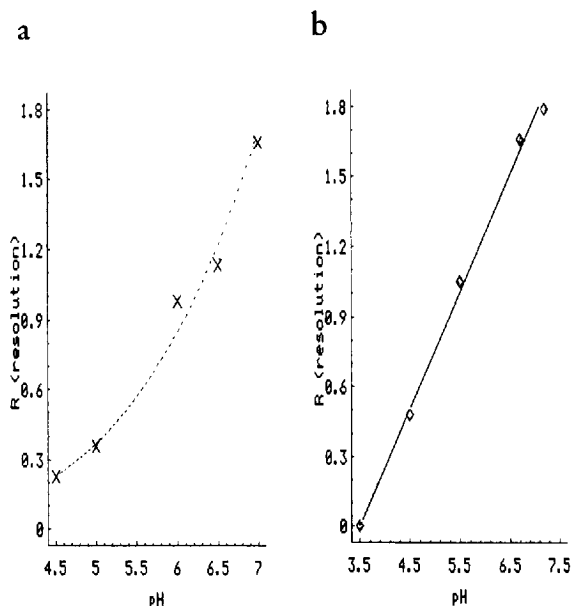

Fig. 4. Dependence of resolution (R_s) of ZP enantiomers on pH using ACN (a) and MeOH (b) as organic modifier.

depends on the shape, size and spatial geometry of the solute, the diameter of the CD cavity and other factors. One of the most important factors affecting inclusion complex formation and chiral discrimination is the relative size of the solute with respect to the size of the CD cavity. Thus, the six compounds under study can be classified into three groups of structurally related compounds.

RP 26695 is a relative small molecule and is expected to form the least stable inclusion complex within the β-CD cavity; in accordance with this theoretical concept the RP 26695 is the first compound eluted after an injection of a mixture of the six solutes under study.

RP 29307 and RP 48497 are potential degradation products after carbamate hydrolysis. Their molecular
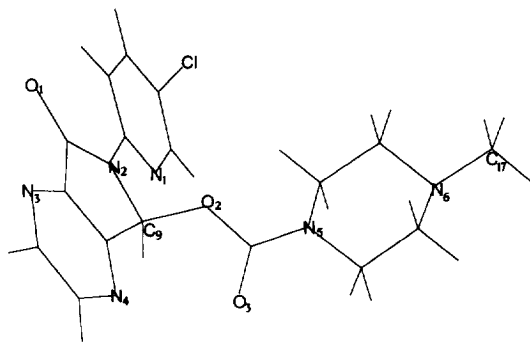
Fig. 6. Crystallographic structure of zopiclone. C(9)–N(3): 3.60 Å, C(9)–C(17): 7.56 Å, C(9)–Cl: 6.58 Å, C(10)–Cl: 10.27 Å.

structure is characterized by the absence of the piperazinyl group which is the only group affected by a decrease of pH. Consequently, the inclusion of these compounds within the β-CD cavity is only slightly affected by a change in pH. As shown in Fig. 7a, the difference between the retention times of RP 29307 and RP 48497 is 10 min; this behavior can be rationalized in terms of the presence of a –OH group in the lactam ring of RP 29307 which makes it more hydrophilic.

The third group comprises the racemates of OXZP, DMZP and ZP. It is important to mention that at pH greater than 6.7 the enantiomers of ZP are eluted after those of its chiral metabolites and at pH less
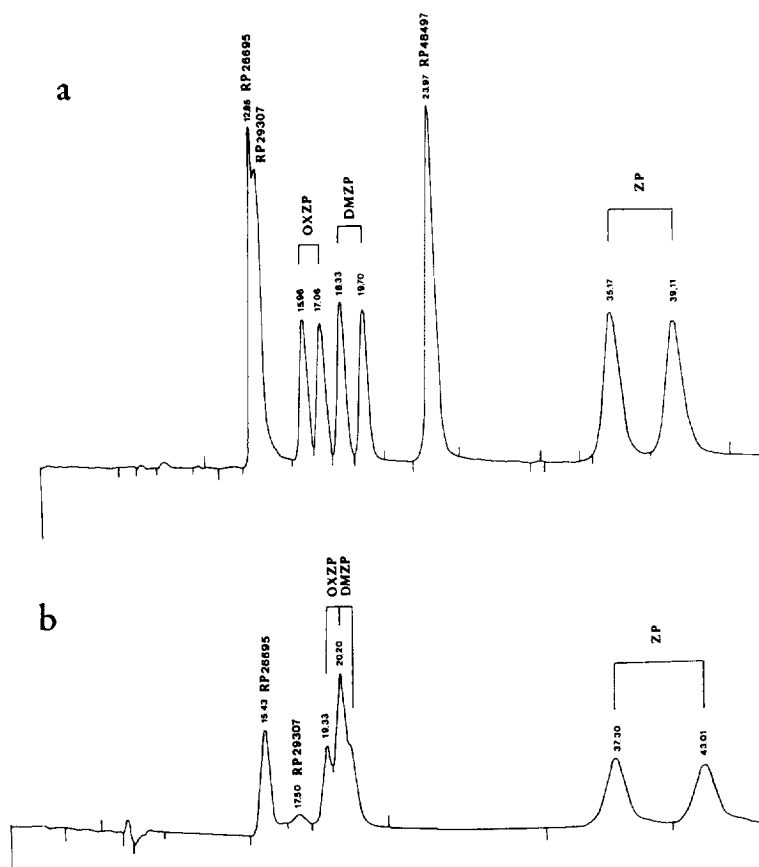

Fig. 7. Representative chromatograms after injection of a mixture of the compounds under study using ACN (a) and MeOH (b) as organic modifier. Chromatographic conditions: buffer–organic modifier 95:5; buffer, 0.5% TEAA (w/v); pH 7.2; flow-rate, 0.5 ml/min.
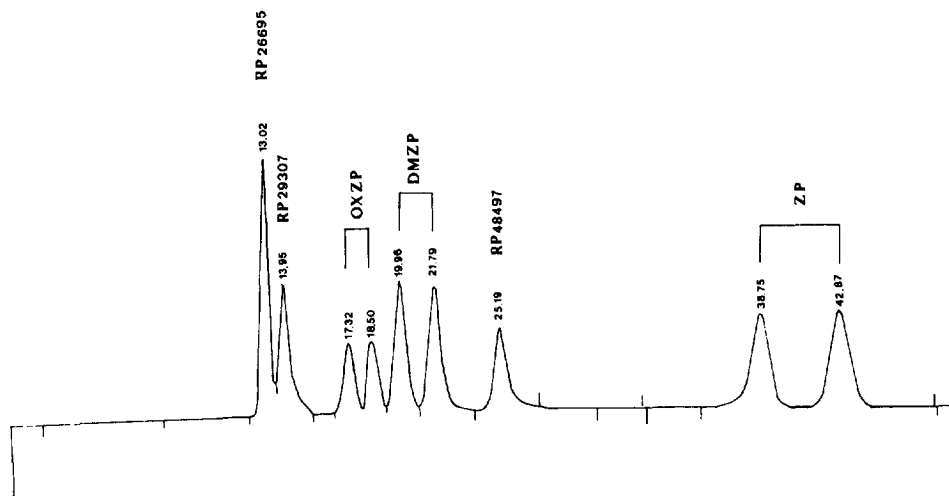
Fig. 8. Representative chromatogram after injection of a mixture of the compounds under study. Chromatographic conditions: buffer–ACN 97:3; buffer, 0.5% TEAA (w/v); pH 7.2; flow-rate, 0.5 ml/min.

than 5.5 are eluted before them; this behavior can be explained as discussed before by the considerable protonation of ZP at pH less than 5.5.

Further, since OXZP is a N-oxide, which is assumed to be more hydrophilic than DMZP, it is expected to be less retained; therefore, in all studies performed OXZP enantiomers were eluted before those of DMZP.

Several requirements have to be met for chiral recognition to occur in a CD bonded phase. First an inclusion complex should be formed between the solute and CD which can provide a relatively 'tight fit' between the hydrophobic part of the solute and the CD cavity. In addition, the chiral center or a substituent attached to the chiral center must be near and interact with the 'mouth' of the CD cavity [24].

As shown in Fig. 6, based on the crystallographic data of zopiclone [20], a computer model of the molecule has been designed using the program HyperChem. In the zopiclone molecule the distances from the chiral center C(9) to the N(3) of the pyrazinyl group, to the C(17) of the piperazinyl group and to the Cl of the pyridyl groups are 3.60 Å, 7.56 Å and 6.58 Å, respectively. Thus, the molecule possesses three hydrophobic regions whose size permits interactions with the β-CD cavity (internal diameter 7.8 Å) in such a way that the chiral center will be found at the rim of the cavity. On the other hand, the N(1), N(2), O(1), O(2) and O(3) can

participate in at least two possible hydrogen bond interactions with the –OH groups at the circumference of the cavity. These findings are in agreement with the three-point attachment concept initially introduced by Dalgliesh [25].

Another important observation is that in all experiments performed a better resolution was achieved for the enantiomers of ZP than for those of OXZP and DMZP, e.g., under the chromatographic conditions presented in Fig. 7a the resolutions of the enantiomers of ZP, OXZP and DMZP are 1.79, 1.10 and 1.37, respectively.

3.5. Optimization of the chromatographic separation

Generally, the use of ACN as organic modifier leads to a better separation of the enantiomers and the compounds under study than the use of MeOH. Fig. 7 shows representative chromatograms under identical chromatographic conditions using ACN and MeOH as organic modifier.

Further, it seems that by decreasing the flow-rate the molecules have greater opportunity to interact with the β-CD and thus the selectivity of the column increases. Consequently, as the flow-rate decreases both retention and resolution increase.

After investigating the liquid chromatographic retention behavior of all the compounds under study

on a β-CD with respect to mobile-phase composition, pH, ionic strength, and solvent selectivity, a set of chromatographic conditions was chosen for the simultaneous separation of ZP, OXZP and DMZP enantiomers in the presence of potential degradation products, i.e., RP 29307, RP 48497 and RP26695. These conditions are as follows: acetonitrile–buffer, 3:97 (v/v); buffer, 0.5% TEAA (w/v); pH 7.2; flow-rate, 0.5 ml/min. These conditions are proposed for an efficient chromatographic separation of a mixture of these enantiomers and related substances using UV detection (Fig. 8).

Acknowledgments

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